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SEED TESTING FOR BLACKLEG OF CANOLA

P. D. Kharbanda
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Alberta
ENVIRONMENTAL CENTRE

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SEED TESTING FOR BLACKLEG OF CANOLA

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by

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SUMMARY

Blackleg, caused by the fungus *Leptosphaeria maculans*, is a destructive disease of canola in temperate regions of the world. In Alberta, the disease has rapidly spread throughout east-central region after appearing in one field near Vermilion in 1983. Since the fungus can be carried long-distances with infected seeds, growing disease-free seed is an effective way of preventing it from entering into uninfested regions.

This manual gives methodology to test canola seed for blackleg infection. Step by step procedures have been described to isolate and recognize *L. maculans* from canola seed and plant tissue and to identify its virulent strain which is mainly responsible for causing economic losses in the crop. Different seed testing methods have been reviewed to assist laboratories choose the method most suitable under their operative conditions. To familiarize technicians with the disease, a brief description of symptoms of blackleg and the life cycle of the fungus, *L. maculans*, are also presented. Recipes of different culture media, the use of haemocytometer, a glossary of scientific terms used, and various laboratories offering seed testing in Alberta are appended.

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1 INTRODUCTION

This manual on seed testing for blackleg of canola was first prepared for a workshop held in October of 1988 at the Alberta Environmental Centre, Vegreville. The Workshop was organized in response to requests from several seed health testing laboratories for assistance in improving their technical ability to recognize virulent blackleg (*Leptosphaeria maculans L.*) in canola seed. Because of the rapid spread of this disease in northeastern Alberta, the number of farmers in Alberta wanting to get their seed tested for blackleg has been increasing. The disease spread from one known field in 1983 to almost 90% of fields surveyed in 1988 in Region 4 (Kharbanda *et al.*, 1989); however, a large portion of Alberta, including the Peace Region, is still blackleg-free (Evans *et al.*, 1991).

Blackleg is commonly seed-borne, and this is almost certainly the manner in which it initially entered western Canada. The most likely way the disease could enter the Peace Region is with infected seed which places a responsibility on seed growers to have their seed tested for freedom from blackleg. There is also a possibility that Agriculture Canada could establish a zero tolerance for *L. maculans* in canola for the seed marketed in Canada; this would put a greater demand on the seed health testing laboratories for blackleg testing.

The blackleg fungus exists in western Canada in two forms: weakly virulent and virulent (severe or highly virulent) strains (McGee and Petrie, 1978). It is the virulent strain which causes substantial damage to canola crops. These strains may look similar in culture and can only be differentiated by using specialized tests.

The objectives of this workshop were: familiarization with the blackleg disease, its symptoms on different plant parts, and methods of disease spread; isolation of *L. maculans* from canola seed, its identification and differentiation from other common canola seed-fungi; and differentiation between weakly virulent and virulent strains of *L. maculans*.

There are several limitations with existing seed-testing methods for blackleg. The 2,4-D blotter method recommended by the International Seed Testing Association (Maguire, *et al.*, 1978) has limited use since 2,4-D sodium salt is not registered as a herbicide in Canada and is not available here. Moreover, it is difficult to reliably differentiate between virulent and weakly virulent strains with this method alone, and it must be supplemented with other tests. Other reliable immunological, cultural and plant inoculation methods are available but are time

consuming or quite complicated and require very specialized equipment not usually available in most seed testing labs.

This manual was written with those technicians in mind that have very limited knowledge of plant pathology. Therefore, different methodologies are described in detail. Several colour photographs are presented to show different characteristics of the weakly virulent or virulent strains of *L. maculans*, and to show the disease symptoms on canola seed and plant. The choice of tests by individual laboratories will largely depend upon the facilities available and the accuracy desired.

2 BLACKLEG OF CANOLA: THE DISEASE

2.1 Importance

Blackleg caused by *Leptosphaeria maculans* (*Phoma lingam*, conidial state) is a destructive disease of canola and has inflicted serious losses to cruciferous crops around the world. In Saskatchewan, where the disease was first recorded in 1975, the annual crop losses are estimated at 10% to over 50% resulting in revenue losses of $20-30 million. Severe annual economic losses have been reported from Alberta, Manitoba and Ontario since 1988. The fungus has a wide host range and attacks several members of the family Cruciferae, including cabbage, cauliflower, mustard, rutabaga, and wild mustard. The disease is also referred to as phoma, dry rot, or stem canker, and is capable of causing seedling blight.

In Canada, two strains, weakly virulent and virulent (also referred to as highly or severely virulent in the literature), have been identified. The weakly virulent strain is not very aggressive and does not cause much damage to the crop. The virulent strain appears early in the growing season and could cause total loss of the crop.

The organism survives from season to season as dormant mycelium in infected seed or infested host tissues.

2.2 Symptoms

The disease affects all plant parts (Plates 1-4). It appears as deep cankers or lesions peppered with black pycnidia, the fruiting bodies of the fungus, on stem base, leaf or pod. The lesions usually have a black margin. The affected plants may be killed or ripen prematurely resulting in poor seed yield. The affected seeds may be shrivelled or discoloured, and may bear pycnidia or show no external symptoms.

2.3 Disease Development and Spread

In spring, the infection can be carried above ground in the cotyledons from infected seed. Lesions formed on cotyledons develop pycnidia which exude a mucilaginous ooze containing pycnidiospores that are carried to neighbouring plants with wind and rain splashings. All infected seeds, however, do not give rise to infected seedlings and the percent seed transmission is quite low, perhaps about 0.5%. Depending upon host susceptibility and suitable environmental conditions, even this mode of transmission could result in serious disease levels. Infected seeds are a principal means of long-distance spread of the disease.

In the field, infested stubble is a major means of disease carryover, and subsequent spread, from one season to the next. Infested host tissue provides inoculum not only in the form of pycnidiospores, but also as ascospores formed in pseudothecia, the perfect state of the fungus. Under moist conditions, millions of ascospores are discharged from pseudothecia and may be carried with the wind to neighbouring canola fields.

Wet and moist weather conditions and temperatures between 18-23°C, especially during early stages of crop growth, are quite conducive to the development of severe disease.

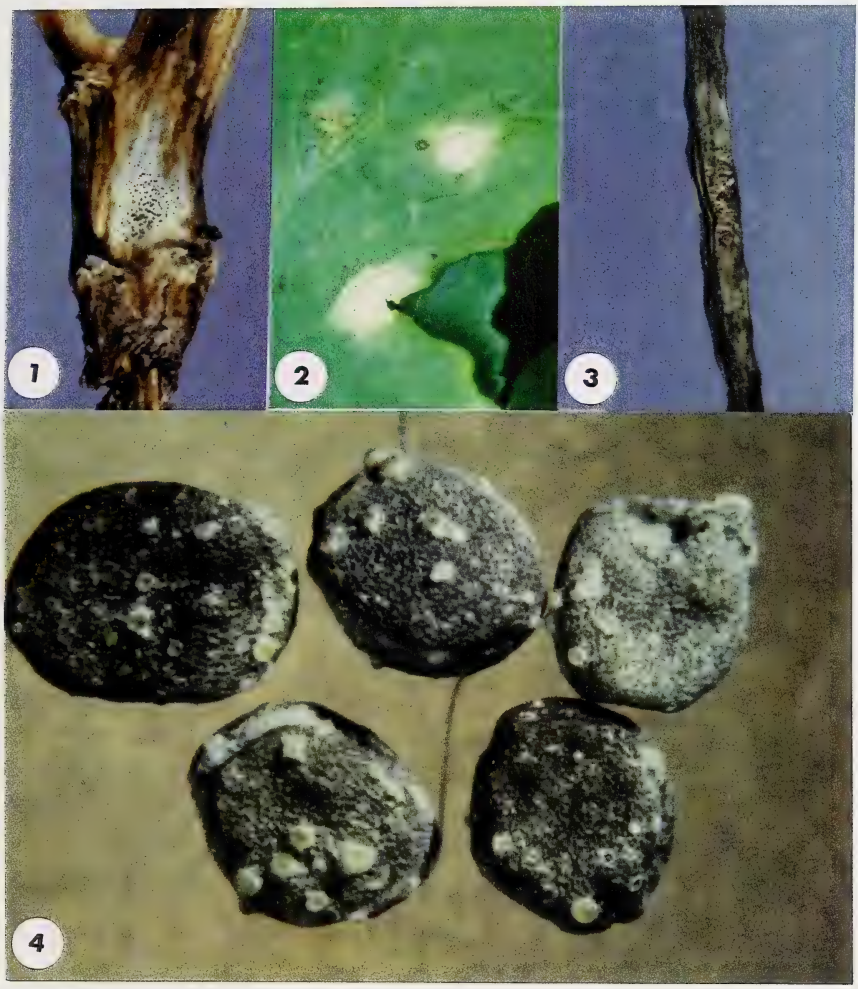


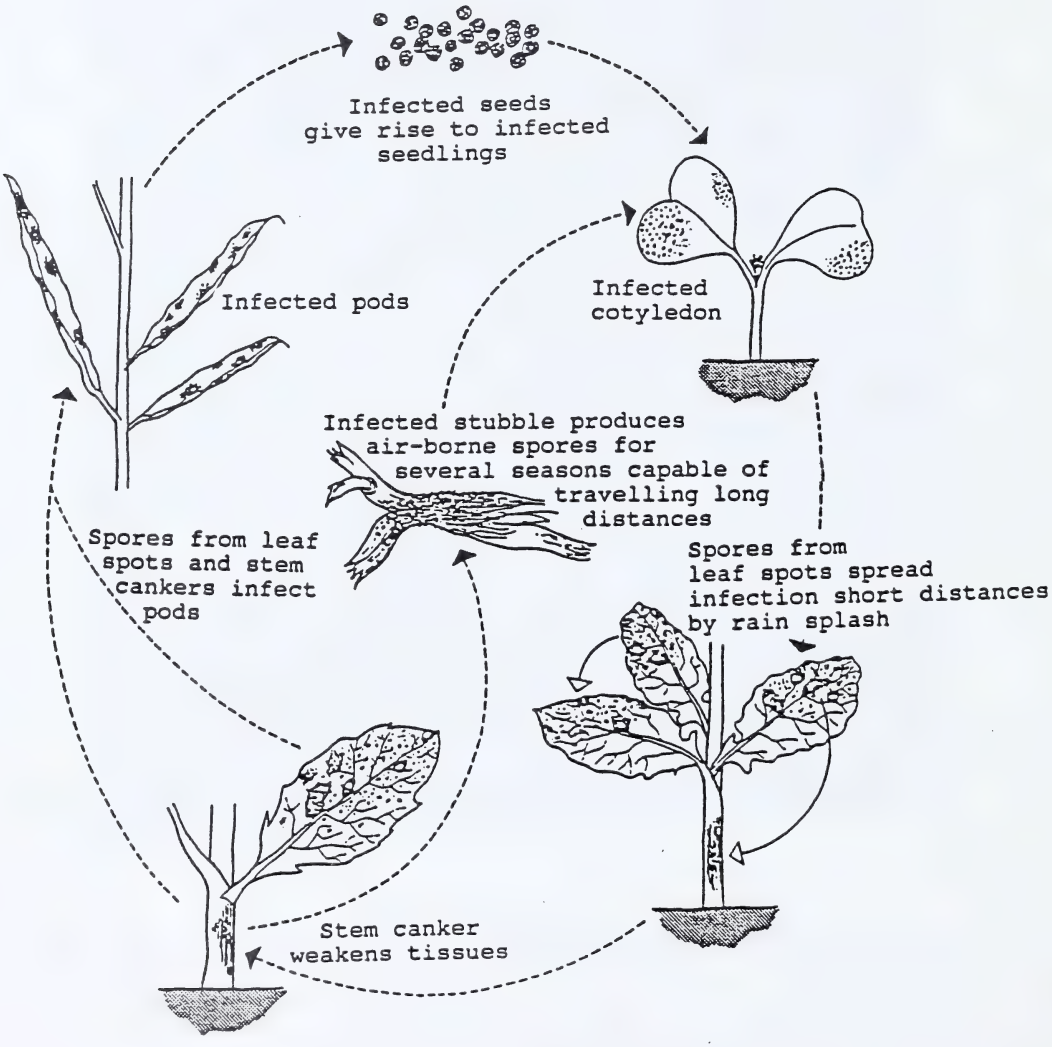
Plate 1. Canola crown lesion with dark pycnidia and root rot caused by *Leptosphaeria maculans*.

Plate 2. Typical early leaf lesions with pycnidia caused by *L. maculans*.

Plate 3. Canola pod infected by *L. maculans*. Pink, spore-containing ooze is visible on the surface of the infected area.

Plate 4. Pycnidia of *L. maculans* formed on infected canola seeds.

2.4 Blackleg Disease Cycle



3 TESTING SEED FOR BLACKLEG INFECTION

Canola seed can be tested for blackleg infection in two ways:

3.1 Isolating, and culturing, the fungus from the seed using selective media such as V8RBS or PDA (Appendix A).

3.2 Inducing pycnidia production on the seeds on moist, sterile filter papers.

Both methods are equally good. However, if the virulence test is to be performed, the fungus recovered on a culture medium can be stored for several months and the test for virulence postponed and performed later. On the other hand, the pycnidia developed on seed in the filter paper test cannot be stored for more than a week or two.

3.1 Isolation and Culture of the Fungus

3.1.1 Seed Disinfestation and Plating

Surface sterilize the seed before plating on a culture medium or filter papers to eliminate the many surface contaminants that make isolation of seed-borne fungi difficult.

Soaking in a 1% sodium hypochlorite (NaOCl) solution for two minutes is the most common method of surface sterilizing seed. Dilutions of household bleach can be used but check the percentage of sodium hypochlorite on the bleach bottle as this can vary. Dilutions can be made using ordinary tap water, but sterile water is preferable.

Small seed such as canola can be surface sterilized in a wire mesh tea-ball. When the seed is immersed in the chlorine solution, shake the tea-ball to dislodge any air bubbles and ensure the chemical is in contact with each seed.

After surface sterilizing for the required amount of time, rinse the seed by draining the tea-ball and placing it in a beaker of sterile distilled water.

Empty the seed onto several layers of sterile filter paper in a sterile petri dish. Replace the petri dish lid and shake the seed to remove excess moisture. The seed should not be plated until it is almost dry.

Use sterile forceps to place the seed on the agar plates or filter paper. Sterilize the forceps by dipping the ends in 95% ethyl alcohol or methanol, passing them through a flame and allow to cool for a few seconds. Repeat this after every 10 seeds or so (about one plate). If a

vacuum-plate is used to pick up the seeds, wipe the face of the plate with alcohol and allow it to air dry.

If there is a problem with contamination by saprophytic fungi, try a longer seed-soak (10 minutes in 0.5% chlorine solution). Also, instead of using a tea-ball, place the seeds in chlorine solution in a filtering flask with a sideneck; create, and break low vacuum in the flask 3-4 times during the seed soaking period. This procedure is helpful in dislodging air bubbles and fungal spores from seed surface but there is also a risk of killing the blackleg fungus present just under the seed coat.

3.1.2 Preparation of Artificial Culture Media

Prepare all media according to directions on the container. Consider the following points.

Sterilize all media in an autoclave at 121°C, 20 lbs pressure for 20 minutes unless specified otherwise.

Cooling all agars to 55°C prior to pouring into plates reduces the amount of condensation on the underside of petri plate covers and also enables the flasks to be handled without discomfort.

Media to which antibiotics are to be added must be first cooled to 55°C as high temperatures inactivate the antibiotics.

Antibiotic solutions can be sterilized by passage through a sterile filter system (0.22 micron cellulose acetate membrane).

If proper media dispensing facilities are not available (e.g. laminar-flow hood or automatic petri dish pourer), pour plates on a clean bench away from air circulation vents and in close proximity to an open-flame bunsen burner.

Slowly passing the lip of the flask through a bunsen burner flame after pouring several plates will help avoid contamination.

Do not open the presterilized dishes any more than necessary when pouring in the agar.

Allow the agar to set for several hours or overnight, then store plates upside down in sealed plastic bags at 4°C.

3.1.3 Isolation of *L. maculans* from Canola Seed

Place surface sterilized (see earlier) seed, evenly spaced, 10 seeds per plate on V8RBS or PDA using sterile forceps.

Carefully stack and bag plates, and incubate at room temperature for 3-4 days.

Select plates showing fungal growth and place them under NUV lights (sporulation chamber) for 2-3 days to promote spore production.

Fungal colonies of *L. maculans* (*Phoma lingam*) are whitish with somewhat fluffy mycelium embedded with round, pin-head type, brownish-black structures called pycnidia which produce pycnidiospores, (Plates 5, 6). Sometimes, the fungal mycelium has to be teased away to see the embedded pycnidia. When observed from underneath the petri plate or using transmitted light, the pycnidia look like small dark brown dots.

Pycnidiospores are unicellular, elliptical and hyaline. They are extruded from the opening (ostiole) of the pycnidium in a pinkish, creamy material.

If cultures are clean, transfer suspected *L. maculans* isolates to fresh PDA plates, otherwise proceed with culture purification techniques.

Proceed to virulence testing with pure cultures. Not all cultures from each sample need to be checked for virulence, however, cultures producing pycnidia and having different growth rates or gross morphologies within the same sample should be checked. Both the virulent and weakly virulent strains of the fungus can be present in the same field or sample.

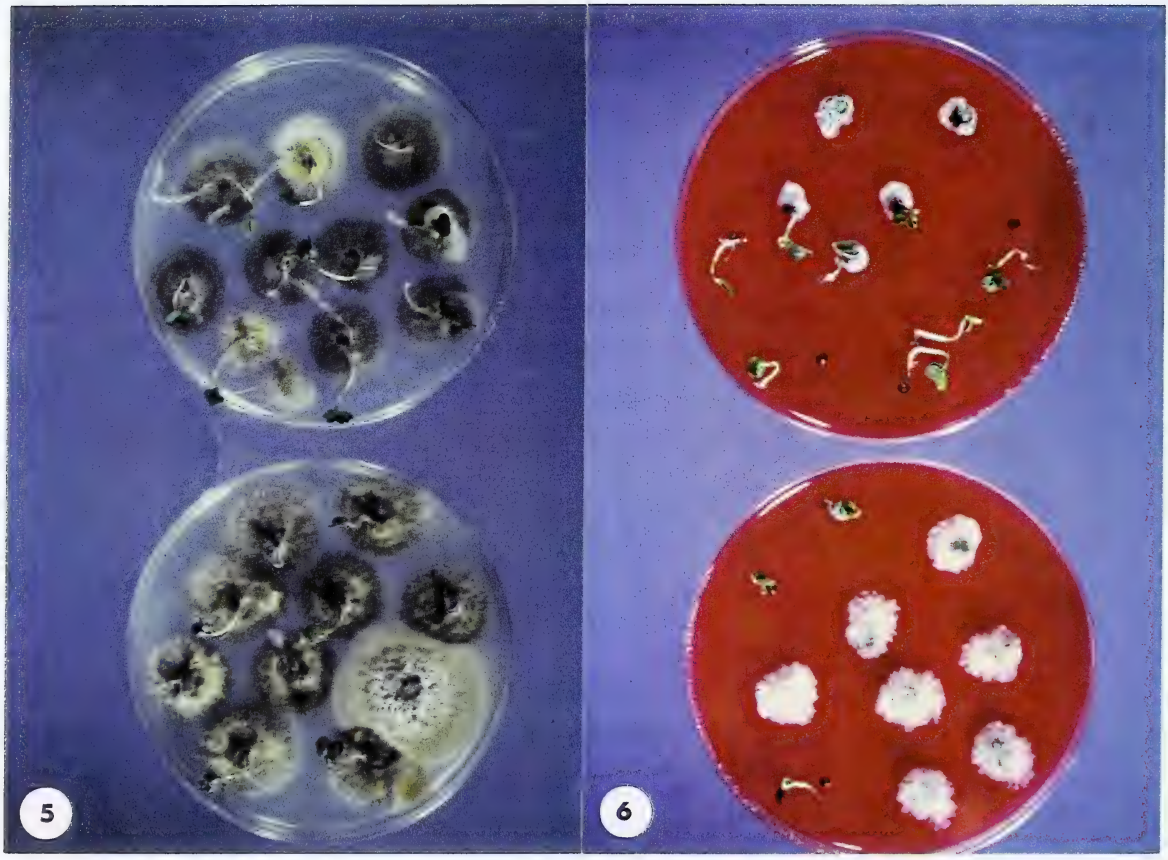


Plate 5. Canola seed artificially infested with virulent *L. maculans* (top) and weakly virulent *L. maculans* (bottom) on PDA 12 days after plating.

Plate 6. Canola seed artificially infested with virulent *L. maculans* (top) and weakly virulent *L. maculans* (bottom) on V8RBS 12 days after plating.

3.1.4 Purification of Fungal Isolates

Before the virulence can be checked, the suspect isolates must be pure cultures. Often good surface sterilization of the seed is all that is needed to get pure cultures but sometimes the colonies which grow from the seeds will be contaminated with bacteria and/or other fungi.

Plating the seed on a selective medium such as V8RBS will help to control some of the contaminating fungi and will suppress the growth of bacteria. Unfortunately V8RBS also seems to inhibit the growth of the target fungus *L. maculans*.

Where bacterial contamination is a problem, the cultures can be transferred onto PDA.A or PDA containing 200 ppm streptomycin sulphate and, when the fungus has grown enough on this medium, transferred back onto PDA or to CZY.

Fungal contaminants are harder to overcome and if you are unable to transfer from an area of the colony not overgrown by the contaminating fungi, the only method of getting a pure culture may be by transferring single spores. Most common contaminants grow faster than *L. maculans*, therefore, occasionally, isolating single spores may be difficult.

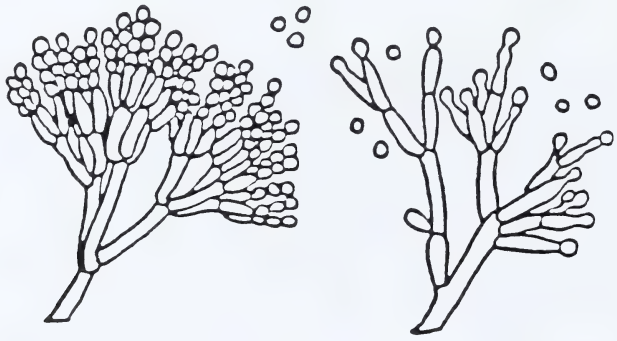
To make a single spore culture of an isolate, place a small amount of pycnidial ooze into a sterile culture tube containing about 10 mL of sterile distilled water. Shake vigorously to disperse the spores and pour the suspension onto a WA plate. Swirl the plate a few times and pour the excess suspension onto another WA plate. Repeat until all the liquid is used or until you have four plates. Incubate the plates on a slant at room temperature and check at 24 and 48 hours for germinating spores. The spores are very small but can be seen under a dissecting microscope at X100. Germinating spores will have short germ tubes which look like tails. Locate spores which are widely separated, pick them off and transfer to PDA. Make 5 or 6 plates of each isolate to be sure you get one good one.

3.1.5 Common Contaminants

Several fungi other than *L. maculans* might be isolated from the seed (Plate 7). Only those which we find most commonly will be discussed here. None of them produce pycnidia and they are all easy to distinguish from *L. maculans*. These fungi are:

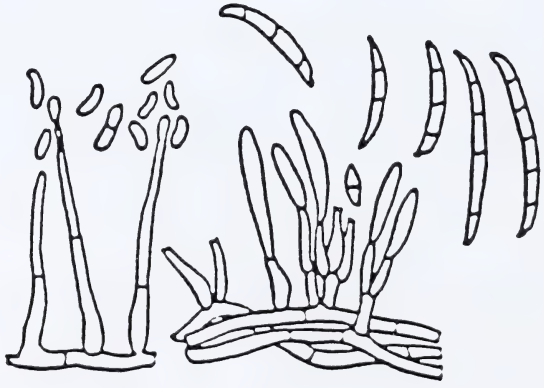
3.1.5.1 *Penicillium* spp.

Colonies are usually blue or green and powdery in appearance with a white outer margin. The conidia (spores) are small, round and one-celled. They are easily dislodged from the spore bearing conidiophores. On examination under the microscope, the typically arranged phialides on the ends of the conidiophores are easily recognized.



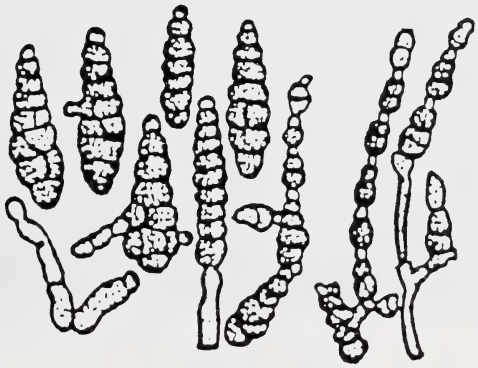
3.1.5.2 *Fusarium* spp.

Colonies are usually very fluffy, white to pink and often red on the underside of the PDA. Conidia may be oval one-celled or distinctly canoe-shaped with several septa. The conidiophores are not always easily seen but the gross morphology of the colony and the distinctive shape of the usually abundant and easily formed conidia make this fungus easy to identify.



3.1.5.3 *Alternaria* spp.

Colonies are grey to olive green and get much darker as conidia are formed. Club-shaped, olive coloured conidia are easily recognized. They are borne in chains on short simple conidiophores. The spores are large enough and distinctive enough to be seen in chains using a dissecting microscope at high power. The colonies start out pale grey or olive and could possibly be confused with *L. maculans* at the outset but *Alternaria* grows faster and the mycelium is more dense.



3.1.6 Storage of Fungal Isolates

To store cultures, transfer them to a desired number of PDA tubes. If screw caps are used, only close them part way. Wrap a strip of parafilm around the top of the tube and after incubating the tubes at room temperature for a few days, to ensure the transfers are growing and producing spores, place them in a cooler at 4°C until needed.

We have found that storage of cultures in liquid nitrogen is a superior method for preservation over six months.

Plate 7. Blackleg and common contaminants on PDA after 5 days. Clockwise from the top left: *L. maculans*, *Penicillium* spp., *Fusarium* spp., and *Alternaria* spp.

3.2 Production of Pycnidia

A 2,4-D blotter method (described below) has been recommended by the International Seed Testing Association to determine the presence of *L. maculans* in crucifer seed (Maguire, *et al.*, 1978). By visual examination of seeds it is possible to identify seeds with pycnidia (Plate 4) but is not possible to distinguish between virulent and weakly virulent strains. Also, some infested seeds do not show pycnidia on the seed surface and therefore could be missed.

3.2.1 2,4-D Direct Seed Assay (Blotter Test)

Surface sterilize seed for 10 minutes in a 0.1% sodium hypochlorite solution, rinse in sterile water and allow seed to dry.

Place four sterile filter papers in each sterile petri plate and soak with a 0.2% solution of 2,4-D sodium salt. Use only the sodium salt formulation as other 2,4-D formulations are toxic to the fungus.

Drain off excess solution as too much moisture inhibits growth of the fungus. If it is necessary to remoisten the filter papers during the test, use a few drops of sterile distilled water.

Place the seed on the plates using sterile forceps. Optimum spacing is 8-10 cm.

Check seven days after plating for pycnidia using a dissecting microscope and make a final evaluation after 11 days.

Transfer ooze from pycnidia to PDA plates as a backup, and proceed to virulence testing if cultures are clean.

Because 2,4-D sodium salt is not registered for use on crops in Canada and is not available here, a modified blotter test (Agriculture Canada, 1989) is given below.

3.2.2 Modified Blotter Test

A modified blotter test was developed in consultation with Leslie Cree, Agriculture Canada (1989), Ottawa.

Surface sterilize seed in 1.0% sodium hypochlorite solution for 10 minutes.

Place two layers of sterile filter paper in a sterile petri plate. Add sterile distilled water to soak the filter papers.

We found that adding a 200 ppm solution of streptomycin sulphate instead of sterile distilled water helps inhibit bacteria carried in the seed.

Drain off excess water as too much moisture inhibits growth of the fungus. The filter papers can be remoistened during the test with a little amount of sterile distilled water or streptomycin sulphate solution.

Place the seed on plates using sterile forceps. Optimum spacing is 8-10 mm, (Plate 8).

Incubate the plates (seeds) as follows:

1st to 3rd Days: In dark, 20-25°C

4th Day: In freezer, -20°C

5th-10th Days: 12 hours under fluorescent light followed by 12 hours darkness each day, 20-25°C

3.2.2.1 Examination

Use 50-100 X magnification with an incident light source.

Pycnidia are 200-600 µm diameter, in various shapes, some single or compound globules with ostioles, others may be flask-shaped with long necks. The exudate colour varies from white, pink, amethyst to almost red.

A saprophyte, *Phoma herbarium*, is often found on brassica seeds (Boerema, 1964). These pycnidia are superficially formed on the seed coat and are smaller in size, 80-260 µm in diameter.

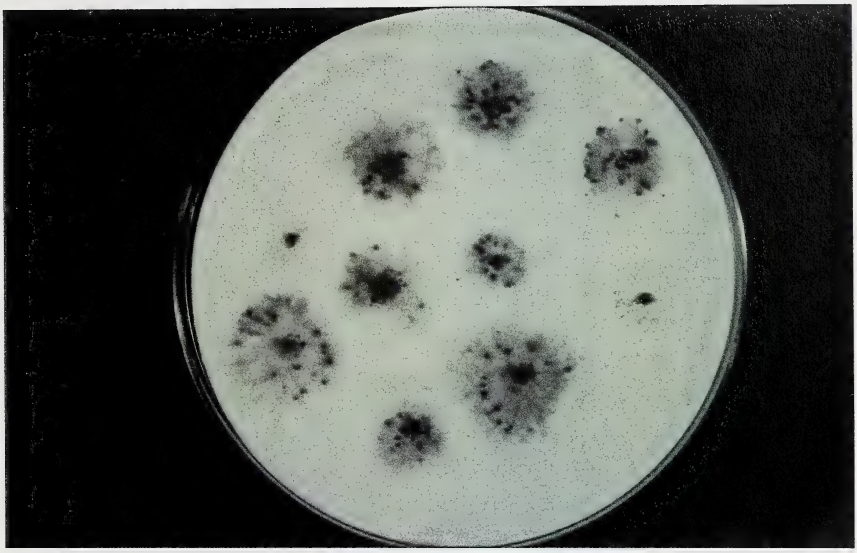


Plate 8. Blackleg fungus growing from infested seeds plated on 2,4-D soaked filter papers after 11 days.

4 TESTING OF VIRULENT STRAINS

The virulent and weakly virulent strains can be distinguished on the basis of any one of the following characteristics; it is suggested that parallel controls of known virulent and weakly virulent strains be included in each test.

4.1 Colony Growth Rate

The virulent strain has a slower growth rate than the weakly virulent strain on various culture media such as potato dextrose agar (PDA) and V-8 juice agar (V8A). We found that colony diameters for several weakly virulent and virulent strains after two weeks on PDA were 50-75 mm and 25-50 mm, respectively (Plate 9).

Prepare PDA or V-8 juice agar. Pour culture plates with about 20 mL medium per plate.

Cut out a small piece of the *L. maculans* culture from the isolate to be tested and also from a known virulent and a weakly virulent isolate. A 4 mm cork-borer works well for this purpose.

Transfer the fungal isolates onto the centre of separate culture plates; prepare at least three plates of each isolate.

Incubate the plates at 20-23°C for 10-14 days. Lights are not necessary.

The weakly virulent strain grows faster than the virulent strain.

This test alone is not sufficient to distinguish between weakly virulent and virulent strains but it does give a very important clue. This test gives the same information as obtained from the Pycnidiospore Germination test described on the next page, but is easier to perform.

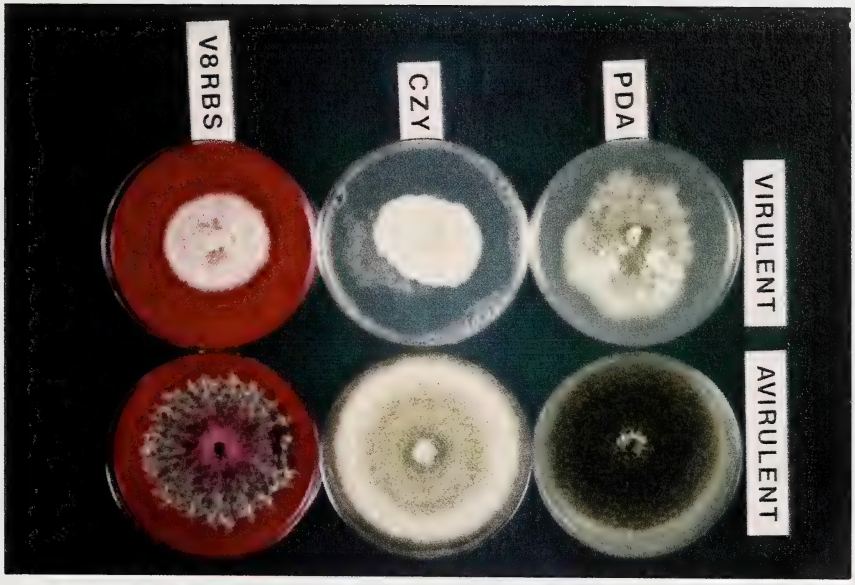


Plate 9. Comparison of colony morphology of virulent (top) and weakly virulent (bottom) blackleg isolates on various culture media two weeks after incubation at 21°C.

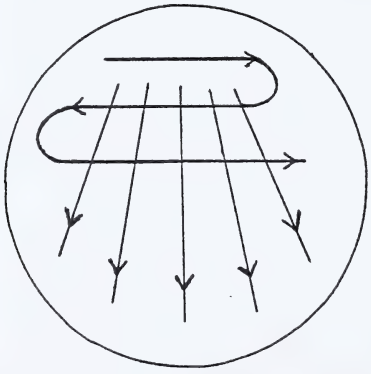
4.2 Pycnidiospore Germination

Recently, Petrie (1988) reported mean pycnidiospore germ tube length of several weakly virulent and highly virulent strains of *L. maculans* on water agar (transferred from V8A); those ranged between 179.4±11.4 and 376.8±7.8 for weakly virulent strains and between 75.6±2.4 and 98.3±8.8 for the highly virulent strains. This method is faster than the one in which we determine colony growth rate but the germinating pycnidiospores have to be observed soon after the 44 hour incubation period.

This method can be used with cultures growing on various media or with pycnidial ooze taken directly from the seeds.

Transfer, aseptically, a minute amount of pycnidial ooze into 10 mL sterile water. If you are using plant material as a source of conidia, surface-sterilize it with 0.5% sodium hypochlorite for 15 minutes, plate on V-8 juice agar, and incubate under UV lights until oozing pycnidia are formed. If oozing pycnidia are present on the plant material, place the material directly into 10 mL sterile water for several minutes.

Remove a drop of spore suspension with a wire loop and streak on 2% water agar containing 100 mg/L streptomycin and 50 mg/L vancomycin. Restreak the agar surface at right angles to the previous streaks to separate the spores as shown below.



Incubate these plates in darkness for 40-44 hours at 20°C.

Measure germ tubes directly viewing the plates under the microscope. Use a magnification of 500X. Measure 10 germ tubes of the clearly separated spores. Germ tubes of virulent strains will be shorter than those of weakly virulent strains (Plate 10).

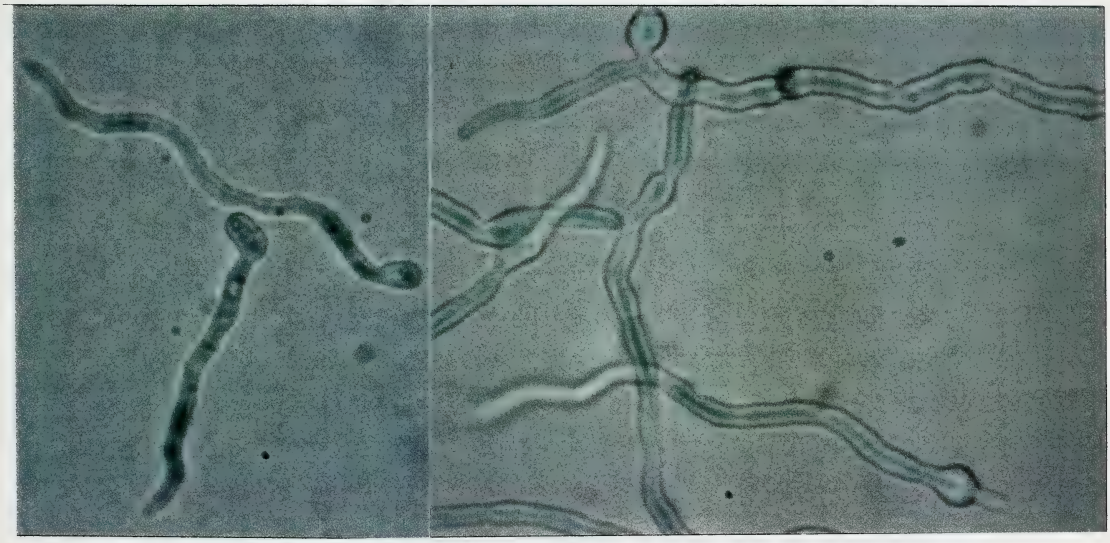


Plate 10. Germinating pycnidiospores of virulent (left) and weakly virulent *L. maculans* on 2% WA with antibiotics after 40 hours (X640).

4.3 Pigment Production on Modified Czapeck Medium

On modified Czapeck agar (CZY), the weakly virulent strains produce pinkish-orange to brown pigment whereas virulent strains do not product any colour (Plate 11) (McGee and Petrie, 1976). The intensity of colour produced may vary between isolates.

Transfer mycelium or pycnidial ooze from pure cultures identified as blackleg on PDA or V8RBS, to CZY (if using V8RBS, avoid transferring the pink agar as this can interfere with the colour change). If the filter paper method was used, transfer pycnidial ooze from plated seeds.

Incubate plates at room temperature for 4-6 days (do not put under NUV light; regular fluorescent light is best).

Check CZY plates for pigmentation. Pinkish orange colour in the agar signifies weakly virulent blackleg. Absence of pigmentation signifies virulent blackleg. The pigment increases in darkness with time and eventually turns dark brown.

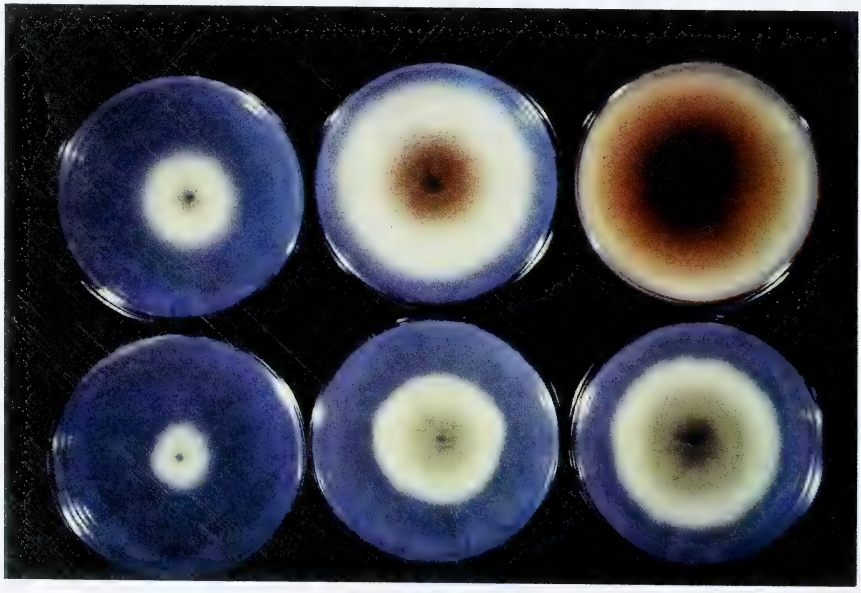


Plate 11. Weakly virulent (top) and virulent (bottom) *L. maculans* on CZY medium showing pinkish orange pigment produced by the weakly virulent isolate after 6, 10 and 14 days (left to right) of growth. Also notice the faster rate of growth of the weakly virulent isolate.

4.4 Pathogenicity on Cotyledons

When inoculated onto seedling cotyledons, the virulent strains invade the plant tissue rather rapidly, producing pycnidia within 7 days (Plate 12). The weakly virulent strains cause darkened necrosis but produce no pycnidia.

Grow canola seedlings until cotyledons are well formed, 7-10 days.

Make a spore suspension of 10^6 spores/mL in 0.5% gelatin solution (Appendix B).

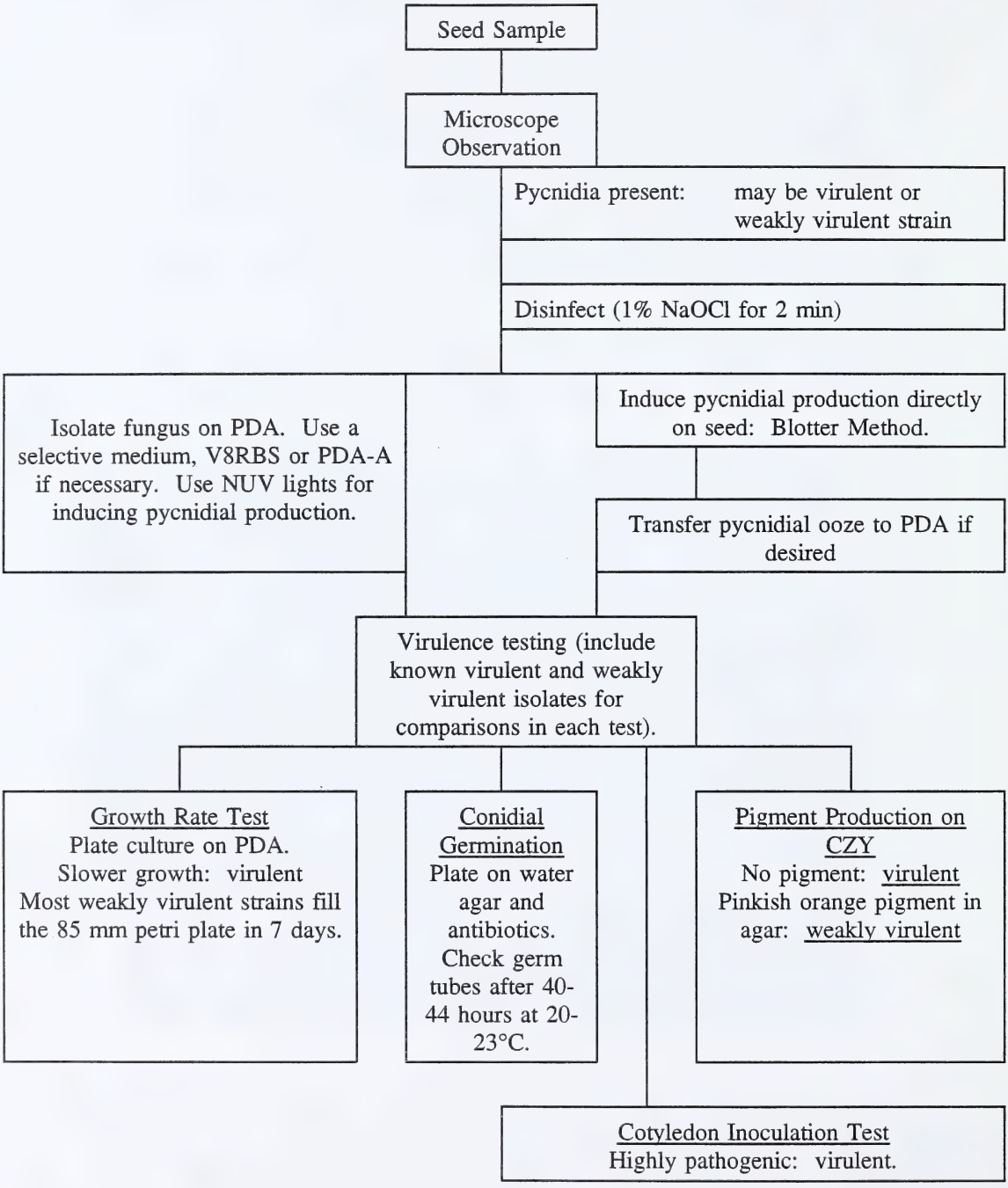
Puncture each cotyledon with a sterile needle and immediately place a small drop of spore suspension over the wound (a small hypodermic syringe is good for this).

Cover pots loosely with plastic bags for 48 hours to increase humidity.

Examine cotyledons after 10 days and evaluate virulence. Weakly virulent isolates will cause slight necrosis (blackening) of tissue immediately around the wound. Virulent isolates will cause larger lesions with extensive tissue collapse along with the formation of pycnidia.

Plate 12. Cotyledons 10 days after inoculation with a virulent (left) and a weakly virulent (right) strain of *L. maculans*. Note extensive tissue damage on cotyledon inoculated with the virulent strain.

5 SEED TESTING FOR BLACKLEG: FLOW CHART


Seed Sample
Microscope Observation
Pycnidia present: may be virulent or weakly virulent strain
Disinfect (1% NaOCl for 2 min)
Isolate fungus on PDA. Use a selective medium, V8RBS or PDA-A if necessary. Use NUV lights for inducing pycnidial production.
Induce pycnidial production directly on seed: Blotter Method.
Transfer pycnidial ooze to PDA if desired
Virulence testing (include known virulent and weakly virulent isolates for comparisons in each test).
Growth Rate Test
Plate culture on PDA.
Slower growth: virulent
Most weakly virulent strains fill the 85 mm petri plate in 7 days.
Conidial Germination
Plate on water agar and antibiotics.
Check germ tubes after 40-44 hours at 20-23°C.
Pigment Production on CZY
No pigment: virulent
Pinkish orange pigment in agar: weakly virulent
Cotyledon Inoculation Test
Highly pathogenic: virulent.

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APPENDIX A

APPENDIX A. RECIPES FOR CULTURE MEDIA

V-8 Juice with Rose Bengal and Streptomycin Sulphate (V8RBS)

V-8 Juice	200 mL
Distilled Water	800 mL
$CaCO_3$	0.75 g
Rose Bengal (4% solution)	10 mL
Difco Bacto Agar	15 g

Autoclave at 121°C, 20 lbs pressure for 20 minutes. Allow to cool to 55°C and add:

Sterile Streptomycin Sulphate (5% solution)	2 mL

Modified Czapeck Medium for Blackleg (CZY)

Distilled Water	1000 mL
$NaNO_3$	2.0 g
K_2HPO_4	1.0 g
KCl	0.5 g
$MgSO_4.7H_2O$	0.5 g
$FeSO_4.7H_2O$	0.01 g
Sucrose	30.0 g
Yeast Extract	2.0 g
Difco Bacto Agar	15.0 g

Dissolve ingredients in distilled water in the order given and autoclave at 121°C, 20 lbs pressure for 20 minutes. (Agar will not dissolve until media is heated or autoclaved.)

Potato Dextrose Agar (PDA)

Difco Bacto Potato Dextrose Agar	39 g
Distilled Water	1000 mL

Autoclave at 121°C, 20 lbs pressure for 20 minutes.

Acidified Potato Dextrose Agar (PDA.A)

To 1 litre autoclaved PDA which has been cooled to 55°C, add 6 mL sterile 25% lactic acid. Mix well before pouring plates. The lactic acid may be autoclaved separately but must not be added to the medium until after the medium is autoclaved otherwise it will prevent the agar from solidifying.

Water Agar (WA)

Difco Bacto Agar	15 g
Distilled Water	1000 mL

Autoclave at 121°C, 20 lbs pressure for 20 minutes.

2% Water Agar

Difco Bacto Agar	20 g
Distilled Water	1000 mL

Autoclave at 121°C, 20 lbs pressure for 20 minutes.

V-8 Agar (V8A)

V-8 Juice	200 mL
$CaCo_3$	2 g
Difco Bacto Agar	20 g
Distilled Water	800 mL

Autoclave at 121°C, 20 lbs pressure for 20 minutes.

APPENDIX B

APPENDIX B. PREPARATION OF CONIDIAL SUSPENSIONS AND USE OF HAEMACYTOMETER

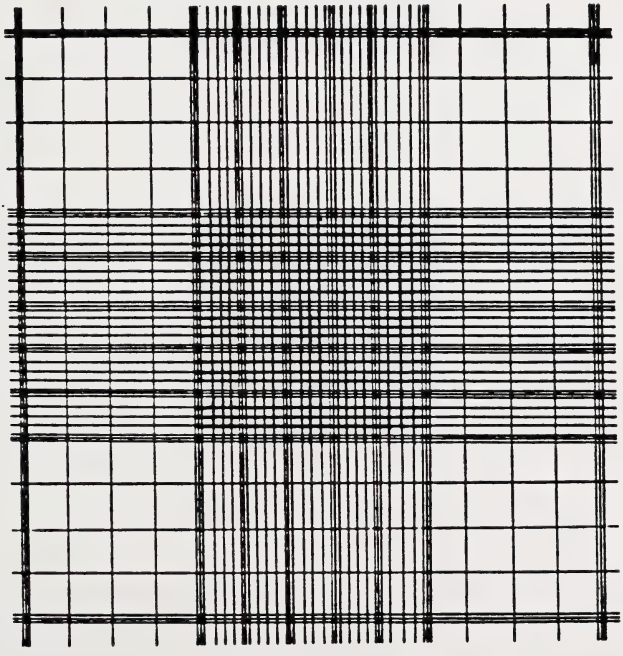
Conidia production by many fungi, including *L. maculans*, can be hastened by placing the cultures in a sporulation chamber. This is an open box, painted silver or white on the inside with one 40 W near-ultraviolet fluorescent lamp and two 40 W cool white daylight fluorescent lamps spaced 20 cm apart and suspended 40 cm above the top of the culture plates. The lights should be connected to a timer to alternate cycles of 12 hours light and 12 hours dark.

To prepare conidial suspension for cotyledon inoculation:

1. Start with a pure culture of the fungus which is producing oozing pycnidia.

2. Flood the culture with sterile 0.5% gelatin solution and rub the surface of the plate with a glass rod to dislodge the spores.

3. Empty the conidial suspension into a sterile beaker and add more 0.5% gelatin solution until the proper spore suspension (approximately 10^6-10^7/mL) has been reached. Determine this by counting the number of spores in the 25 Primary Squares on a haemacytometer. A sketch from the improved Neubauer Bright Line haemacytometer is presented below:



One hundred spores in these 25 squares equals 10^6 spores/mL. Shake the suspension well before withdrawing a sample for the haemacytometer.

APPENDIX C

APPENDIX C. GLOSSARY

ascospores: sexually produced spores borne within a sac-like cell (the ascus, plural asci).

aseptic: free of disease causing organisms.

conidia: asexual spores produced externally on a specialized hypha.

conidiophore: specialized hypha bearing conidia.

germ tube: the hypha produced by a fungal spore when it begins to germinate.

gross morphology: the overall form and structure of an organism; size, color, etc.

hyaline: colorless, transparent.

hypha: a filament of fungal mycelium composed of one or more cylindrical cells.

mycelium: the mass of hyphae constituting the vegetative body of a fungus.

ostiole: the opening of a fungal fruiting body such as a pycnidium or perithecium through which the spores are discharged.

perithecia: flask-like, thick-walled fruiting bodies of some fungi (Ascomycetes), containing sexually produced asci and ascospores.

phialid: the structure at the end of a conidiophore from which spores are produced. The spores are generally produced inside the phialid and extruded.

pycnidia: asexual, thick-walled fruiting bodies lined inside with conidiophores.

pycnidiospores: asexual spores borne on conidiophores inside pycnidia.

septa: cross walls in the hyphae or spores of fungi.

strain: a pure line of an organism.

virulence: relative ability to cause disease; a measure of pathogenicity.

APPENDIX D

APPENDIX D.

ALBERTA LABORATORIES OFFERING BLACKLEG TESTING

Alberta Wheat Pool
4722 - 39 Street
Camrose, Alberta
T4V 0Z5

Contact: Bernice Heck
Phone: 403-672-5571
Fax: 403-672-6971

Alberta Wheat Pool
9728 - 128 Avenue
Grande Prairie, Alberta
T4V 4J4

Contact: Michele Kulba
Phone: 403-532-8842
Fax: 403-539-1924

Norwest Labs
9938 - 67 Ave.
Edmonton, Alberta
T6E 0P5

Contact: Cheryl Engler
Phone: 403-438-5522
Fax: 403-438-0396

United Grain Growers Ltd.
Seed Division
Box 6030, Station "C"
Edmonton, Alberta
T5B 4K5

Contact: Brenda Winnicki
Phone: 403-479-2051
Fax: 403-479-6027

20/20 Seed Labs Ltd.
702 - 21 Avenue
Box 224
Nisku, Alberta
T0C 2G0

Contact: Sarah Foster-Stubbs
Phone: 403-955-3435
Fax: 403-955-3428

Brooks Diagnostics Ltd.
Plant Health Clinic
P.O. Box 1701
Brooks, Alberta
T1R 1C5

Contact: Barbara Penner
Phone: 403-362-5555
Fax: 403-362-5556

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